Trends in Microbiology

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Review

Cellular roadmaps of viroid infection

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Viroids are single-stranded circular noncoding RNAs that infect plants. According to the International Committee on Taxonomy of Viruses, there are 44 viroids known to date. Notably, more than 20 000 distinct viroid-like RNA sequences have recently been identified in existing sequencing datasets, suggesting an unprecedented complexity in biological roles of viroids and viroid-like RNAs. Interestingly, a human pathogen, hepatitis delta virus (HDV), also replicates via a rolling circle mechanism like viroids. Therefore, knowledge of viroid infection is informative for research on HDV and other viroid-like RNAs reported from various organisms. Here, we summarize recent advancements in understanding viroid shuttling among subcellular compartments for completing replication cycles, emphasizing regulatory roles of RNA motifs and structural dynamics in diverse biological processes. We also compare the knowledge of viroid intracellular trafficking with known pathways governing cellular RNA movement in cells. Future investigations on regulatory RNA structures and cognate factors in regulating viroid subcellular trafficking and replication will likely provide new insights into RNA structure–function relationships and facilitate the development of strategies controlling RNA localization and function in cells.

Highlights

More than 20 000 viroid-like RNAs have recently been identified from available high-throughput sequencing datasets, implying that the diversity and function of viroids and viroid-like RNAs are far more complex than currently known.

Viroids replicating in the nucleus use a conserved RNA motif, C-loop, to enter the nucleus, a process that is facilitated by host Virp1 protein and the Importin alpha-4 based pathway.

The active RNA polymerase II (Pol II) complex on potato spindle tuber viroid RNA templates has a distinct organization as compared with the 12-subunit Pol II complex on DNA templates.

Dynamic alterations of viroid RNA structures govern specific processes during viroid infection. For instance, the change from the hairpin I structure to the formation of loop E motif facilitates viroid RNA cleavage and ligation.

Introduction

Viroids are exogenous single-stranded circular noncoding RNAs that replicate in plants. Notably, viroids do not have a DNA phase in their life cycles. There are two viroid families: *Pospiviroidae* and *Avsunviroidae*. Members of the two families differ mainly in their genome structures, replication cycles, replication sites, and the presence or absence of ribozyme activity [1–3]. Currently, there are 39 formal members of *Pospiviroidae* [4] and five formal members of *Avsunviroidae* [5].

The first viroid, potato spindle tuber viroid (PSTVd), was reported in 1971 [6]. Identification of viroids in the early days relied on polyacrylamide gel electrophoresis detecting highly accumulated circular RNAs in infected samples [7]. With the development of high-throughput sequencing (HTS), the first bioinformatic tool for identifying viroids and viroid-like RNAs was developed by analyzing viroid-derived small RNAs [8]. Later, various bioinformatic tools became available for high-throughput discovery of viroid-like sequences from sRNA-Seq or RNA-Seq datasets [9]. A key criterion for establishing a new viroid is whether the RNA can infect any hosts. For example, citrus transiently-associated hammerhead viroid-like RNA1 (CtaHVd-LR1) was identified in an RNA-Seq dataset from a citrus sample, but it was not infectious to citrus through grafting, and it was not found in the same citrus source in the following years [10]. Therefore, CtaHVd-LR1 is not a confirmed viroid species. Recently, thousands of distinct covalently closed circular RNA sequences (i.e., viroid-like RNAs) with structural similarities to known viroids were identified from a broad range of samples, indicating that viroid-like RNAs and their potential functions are far broader than currently known [11–13]. However, the functions of most viroid-like RNAs remain unknown, and their biogenesis mechanisms remain to be determined. Some of the sequences may be retrozyme circular RNAs [14] or retroviroid-like RNAs [15], which are possible endogenous transcripts from retrotransposable DNA elements or pararetrovirus-derived extrachromosomal DNA elements with the potential to amplify via an RNA–RNA pathway [14,16]. Some

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viroid-like RNAs in fugus even act like known viroids to propagate without any DNA phase [12]. Knowledge of viroids may inform future functional studies of the increasing number of viroid-like RNAs.

Multiple attempts have been made to introduce known viroids (i.e., formally recognized species by the International Committee on Taxonomy of Viruses) into yeast [17–19] or even cyanobacteria [20]. Avocado sunblotch viroid (ASBVd), the type species of the *Avsunviroidae* family, was reported to replicate in *Saccharomyces cerevisiae* [17] and a cyanobacterium [20] that lack chloroplasts. Future investigations are needed to help interpret these observations. In another case, alternative processing, but not the complete replication, of PSTVd RNAs was observed in *S. cerevisiae* [18]. Interestingly, a recent report claimed that known viroids may replicate in agriculturally important fungal pathogens [19]. However, the study could not convincingly demonstrate the accumulation of circular progeny using RNA gel blots, a standard experiment in the field. Moreover, known viroids have not been found in hosts other than plants in nature. Therefore, despite the discovery of thousands of viroid-like RNAs in various biological samples, the term viroid is strictly used for formal members of *Avsunviroidae* and *Pospiviroidae*. Hence, whether viroids can naturally infect hosts other than plants remains controversial [21].

In the past 50 years, significant progress has been made in understanding viroid RNA structures, various host factors, the interplay between RNA silencing and viroids, possible mechanisms underlying pathogenesis, etc. [1–3,22–25]. In particular, recent advancements have illuminated viroid RNA structure-based interactions with host machinery for intracellular trafficking and replication. In this review, we provide a timely update on the mechanism underlying viroid trafficking and replication as well as insights into the connections between subcellular movement and replication. Since viroids have genetic information for shuttling among various subcellular compartments, knowledge of viroid trafficking in cells, particularly the regulatory RNA motifs for specific localization, will likely help to develop strategies to accurately control the subcellular localization of other RNAs. This endeavor will benefit future development of RNA-based biotechnologies.

Routes for viroid RNA entering cells

Entering cells is the first step of viroid infection. The plant cell wall represents a natural barrier against viroid infection. Mechanical wounding of plant tissues provides a common route for viroid RNA to enter the cytoplasm [26]. In agricultural settings, the combination of contaminated farming tools and genetically uniform crops facilitates viroid spreading. Interestingly, transmission can occur through underground root systems. For instance, PSTVd RNAs emitted through injured roots remain infectious in the extracellular environment for up to 7 weeks [27]. Tomato plants cultivated hydroponically can uptake infectious PSTVd RNA through their roots with relatively low efficiency [27].

Insects may transmit viroids, but evidence supporting this mechanism is limited and sometimes inconsistent [26,28]. Tomato planta macho viroid was the first confirmed species that can be transmitted from wild reservoirs to tomato plants by aphid (*Myzus persicae*) [29]. But PSTVd transmission by the same aphid relies on transencapsidation with potato leafroll virus [30,31]. A recent report showed that whitefly (*Trialeurodes vaporariorum*) can enhance the transmission of apple scar skin viroid (ASSVd) in the presence of host phloem protein 2 (PP2), which is a known viroid-binding protein [32]. In general, the insect-borne transmission of viroids remains to be further explored to understand the specific insect–viroid combinations as well as the mechanism facilitating the intake and stability of viroid RNA in insects. Some viroids can invade pollen. Therefore, pollination activities, such as those facilitated by bumblebees, can contribute to viroid spreading [26,33]. While there are many well-documented pollen-borne viroids in both families, only a subgroup of them are seed-transmissible [26,34]. For instance, ASBVd and peach latent

mosaic viroid (PLMVd) are both pollen-borne viroids of *Avsunviroidae* and both can be transmitted to seeds via pollination, but only ASBVd is seed-transmissible [26]. It is assumed that PLMVd cannot invade the embryo in seeds [35].

Nuclear import of viroid RNAs

In eukaryotic cells, various molecules need to shuttle between the nucleus and the cytoplasm to coordinate diverse biological processes (Box 1). Once viroid RNAs enter the cytoplasm during initial steps of infection, they proceed to proper organelles for replication (Figure 1, step a). Members of *Pospiviroidae* replicate exclusively in the nucleus [2]. Microinjection assays with fluorescein-labeled PSTVd RNA transcripts facilitated the direct observation of their nuclear import process [36,37]. The nuclear import of PSTVd is relatively fast, with half-maximal signals in the nucleus appearing in about 20 min [37]. Interestingly, PSTVd nuclear import is independent of cytoskeleton as observed in oryzalin- or cytochalasin D-treated cells [36]. Furthermore, nonhydrolyzable GTP and GDP analogs, GTP-γ-S and GDP-β-S, respectively, did not inhibit PSTVd nuclear import [36]. This observation suggests that PSTVd nuclear import is independent of Ran GTPase activity [36]. However, this evidence does not exclude the possibility of an Importin-based pathway for viroid nuclear import, as previous reports have shown that Importin subunits can import cargos into the nucleus independently of Ran GTPase [38,39].

One of the major questions about nuclear import is how viroid RNAs are specifically selected. Prior attempts to elucidate the underlying mechanism did not pinpoint a clear regulatory signal or cellular machinery for viroid nuclear import [36,40]. A recent report showed that PSTVd loop 26 regulates its nuclear import [41]. While only the Watson–Crick (WC) edge is typically used for base pairing in RNA helices, all three edges, including Hoogsteen and sugar edges (Figure 2) may be used in RNA loop motifs [42]. Loop 26 forms a C-loop structure, where RNA bases interact in a highly ordered and specific manner (Figure 2). Disrupting the noncanonical base pairing in the C-loop prevents PSTVd from localizing to the nucleus [41]. By contrast, disruptive mutants of other PSTVd loops (e.g., loops 6, 7, 15, 19, 27, etc.) do not affect nuclear import [43–47]. The C-loop is conserved in most members of *Pospiviroidae*. Not only viroids, the satellite RNA of Q-strain cucumber mosaic virus possibly relies on its C-loop structure for nuclear localization as well [41]. Therefore, the C-loop structure is likely a conserved nuclear import signal utilized by subviral RNAs.

How is the C-loop recognized by cellular machinery? The C-loop in PSTVd overlaps with the previously mapped Virp1 binding site [48]. Virp1 is a bromodomain-containing protein belonging to the GTE gene family [49]. Downregulation of Virp1 impairs PSTVd replication [50]. Interestingly,

Box 1. Cellular machinery for nuclear import

By and large, nuclear localization signals in protein sequences are recognized by Importin alpha (IMPa) subunits. Importin beta (IMPb) subunits often recognize a cognate IMPa with cargos and transport the complex into the nucleus, regulated by Ran GTPase. When Ran GTPase binds with IMPb in the nucleoplasm, the cargo is released from the complex [102]. While some endogenous RNAs, such as rRNAs, tRNAs, small nuclear RNAs (snRNAs), and heterochromatin-associated short interfering RNAs, have been found to enter the nucleus under certain conditions [41], the regulatory mechanism of RNA nuclear import remains poorly understood in most cases.

The nuclear import of snRNAs (i.e., U1, U2, U4, U5) has been studied extensively. snRNAs are transcribed in the nucleus, processed in the cytoplasm, and transported into the Cajal body to form snRNPs [103]. Since these snRNAs are transcribed by Pol II, they possess a 7-methyl guanosine cap at their 5′ end. In the cytoplasm, the cellular Sm protein binds snRNAs to form an Sm ring with the help of the SMN complex. The Sm ring and the SMN complex bring a methyltransferase to hypermethylate the 7-methylguanosine cap to a trimethylguanosine cap, which signals nuclear import [103]. The processed snRNAs with a hypermethylated cap and the Sm ring are imported into the nucleus by an IMPb and an adapter protein Snurportin-1 in animal cells [103].

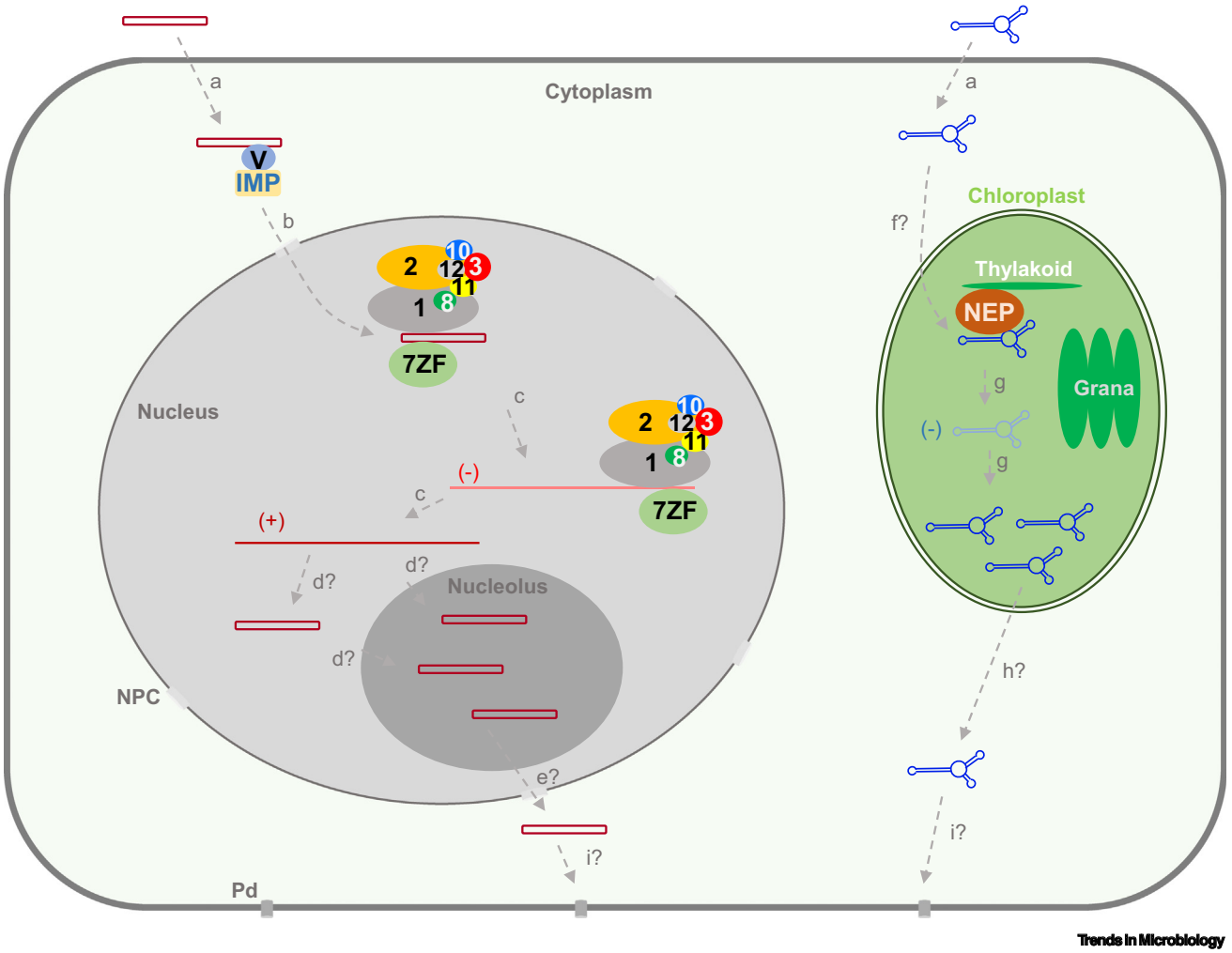


Figure 1. Cellular trafficking of viroids. Illustration of viroid infection routes in cells. Dark and light red lines represent (+) RNA genome of nuclear-replicating viroids of *Pospiviroidae*, respectively. Dark and light blue lines represent (+) RNA genome of chloroplastic viroids of *Avsunviroidae*, respectively. Viroids enter cells through mechanical wounding, pollination, or insects (a). Nuclear-replicating viroids use the C-loop motif to interact with Virp1 (V) and then exploit IMPa-4 (IMP)-based pathway for nuclear import (b) through NPC (nuclear pore complex). In the nucleus, TFIIIA-7ZF (7ZF) aids a remodeled Pol II (a drawing of subunits 1, 2, 3, 8, 10, 11, 12) to catalyze the production of (–) intermediates as well as multimeric (+) intermediates (c). The latter is then cleaved and circularized to generate progeny (d). The progeny is exported out of the nucleus (e). Viroids of the *Avsunviroidae* family enter chloroplasts (f) and engage nuclear-encoded plastid RNA polymerase (NEP) for transcription near the stroma side of thylakoid membrane. The asymmetric replication (g) first generates (–) multimers that are cleaved into unit-length copies via intrinsic ribozyme activity. The unit-length (–) RNAs are circularized by a tRNA ligase to serve as templates for producing (+) multimer RNAs. The (+) multimers are also cleaved by intrinsic ribozyme and circularized by the tRNA ligase to generate progeny. The progeny is then exported to the cytoplasm (h). Viroids use plasmodesmata (Pd) to invade neighboring cells. Question marks indicate that these processes are not well understood.

evidence shows that purified Virp1 can facilitate the import of citrus exocortis viroid (CEVd), a PSTVd relative, into the nuclei of onion cells [51]. Furthermore, Virp1 specifically binds to PSTVd C-loop and this interaction is crucial for RNA localization in the nucleus [41]. Moreover, Virp1 is imported into the nucleus through interaction with IMPa-4, which is an adaptor for the nuclear import of cargos [41]. Therefore, evidence supports that the Importin-based machinery transports the Virp1/viroid complex into the nucleus [41].

Interestingly, mutating PSTVd loop 26 with WC–WC base pairs (loop 26-close) did not impair replication in *Nicotiana benthamiana* protoplasts [52]. In fact, we also observed PSTVd C-loop-disruptive

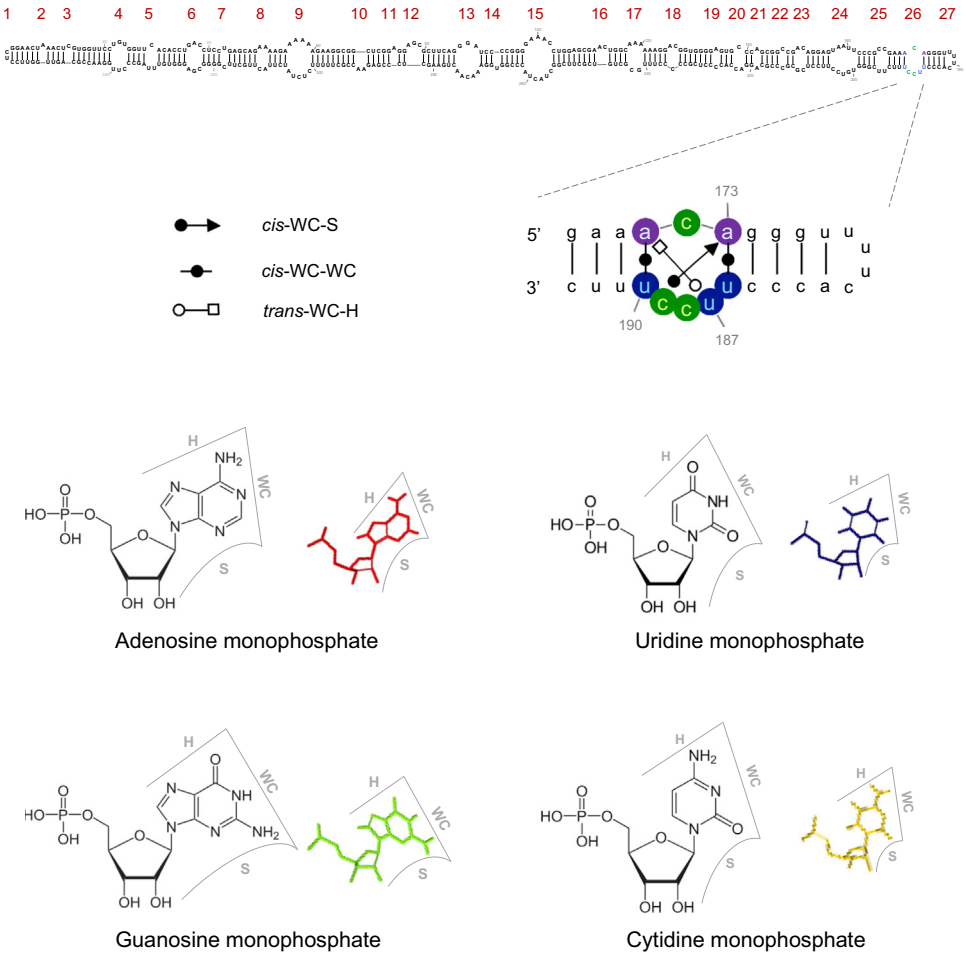


Figure 2. Potato spindle tuber viroid (PSTVd) C-loop structure. The secondary structure of PSTVd genome is shown in the upper panel, with the loop numbers labeled in red. The C-loop is enlarged, with nucleotides therein highlighted in color circles. *Trans* and *cis* refer to the relative positions of glycosidic bonds of two bases in a pairing, respectively. Abbreviations: H, Hoogsteen edge (box shape); S, sugar edge (triangle shape); WC, Watson–Crick edge (circle shape). The three edges (WC, S, and H edges) of each RNA nucleotide are shown in the lower panel (adapted from [42] with permission).

mutants replicating in arabidopsis *(Arabidopsis thaliana).* protoplasts. However, it is important to note that when an arabidopsis protoplast replication assay was performed, a high concentration of calcium was used to potentiate RNA uptake [53]. This abnormally high concentration of calcium is known to interfere with Importin-based nuclear import [54,55]. The replication of loop 26-close mutant was analyzed in *N. benthamiana* protoplasts via electroporation-based transfection [52], which also contains calcium in the electroporation buffer [56]. Therefore, we emphasize here that current protoplast systems are not suitable for studying RNA nuclear import. Nevertheless, these observations support the notion that C-loop only affects nuclear import but not replication.

There are remaining questions regarding the details in the regulation of the IMPa-4/Virp1/Viroid pathway in cells. Nevertheless, a comparison of this pathway with the nuclear import process of snRNAs (Box 1) showed that the C-loop-mediated RNA nuclear import seems simpler in terms of the factors needed and the RNA modifications. This advantage might be helpful for developing a suitable strategy to manipulate RNA subcellular localizations between the nucleus and the cytoplasm.

Pol II-based replication in the nucleoplasm

Unlike RNA viruses that encode their own RNA-dependent RNA polymerases (RdRps) for replication, all viroids must rely on host enzyme(s) to complete the replication process due to their noncoding nature. Pol II is the authentic enzyme catalyzing the transcription of nuclear-replicating viroids by recognizing RNA templates (Box 2). Since Pol II resides in the nucleoplasm, viroid replication most likely occurs in the same compartment (Figure 1, step c).

Pol II is an enzyme that normally functions on double-stranded DNA templates but also possesses intrinsic RdRp activity. This intrinsic RdRp activity can be found in many other DNA-dependent RNA polymerases (DdRps) as well [57–59]. Structural analyses showed that Pol II uses the same catalytic center for both DNA templates and synthetic RNAs containing sequences derived from HDV [60]. An essential question is how Pol II specifically recognizes RNA templates for transcription.

Recent progress has identified TFIIIA-7ZF, a known PSTVd-binding protein [61], as a dedicated transcription factor for PSTVd replication [62]. TFIIIA-7ZF is a splicing variant of transcription factor IIIA (TFIIIA) gene and contains seven C2H2-type zinc-finger domains (ZF). The alternative splicing of TFIIIA is deeply conserved in land plants [63]. Overexpression of TFIIIA-7ZF, but not the other splicing variant (i.e., TFIIIA-9ZF), specifically increases PSTVd titers in plants [62]. Downregulation of TFIIIA transcripts led to inhibition of PSTVd replication *in vivo*. Furthermore, TFIIIA-7ZF, but not TFIIIA-9ZF, drastically increases Pol II-based transcription using circular (+) PSTVd templates *in vitro* [62]. Therefore, TFIIIA-7ZF directly aids Pol II in transcribing the PSTVd RNA genome. The major binding site of TFIIIA-7ZF has been mapped to the lower strand of the left terminal domain, covering loops 3, 4, and 5 [62]. This binding site is in close proximity to the Pol II binding site [64] and transcription initiation site [65] as well as critical for PSTVd replication *in vivo* [52]. Hence, this TFIIIA-7ZF-binding region is considered an RNA promoter [2]. However, the TFIIIA-7ZF-binding region in circular (+) PSTVd can fold into two distinct structures [66]. It awaits to be determined which conformation confers the molecular basis of the RNA promoter [63]. Moreover, whether TFIIIA-7ZF aids the replication of other nuclear-replicating viroids deserves investigations. Interestingly, TFIIIA-7ZF has been shown to interact with hop stunt viroid RNA *in vivo* [63] and to promote the replication of apple fruit crinkle viroid and citrus bark cracking viroid *in planta* [67,68], which supports that TFIIIA-7ZF can be a conserved factor for members of *Pospiviroidae*.

Notably, PSTVd has been found to directly modulate the alternative splicing of TFIIIA transcripts. One intron of the TFIIIA transcript contains a 5S rRNA-mimic structure that is recognized by ribosomal protein L5 (RPL5) to promote intron removal, resulting in the production of TFIIIA-9ZF [69].

Box 2. Evidence supporting Pol II as the authentic enzyme for viroid replication

Since its discovery, there have been multiple attempts to uncover the polymerase responsible for the replication of PSTVd. For example, a DdRp purified from *Escherichia coli* can recognize (+) PSTVd RNA templates to generate dimeric (–) PSTVd products *in vitro* [104]. Similarly, partially purified RdRp from infected tomato samples can also transcribe (+) PSTVd RNA to produce full-length copies *in vitro* [105]. These reports suggest that diverse RNA polymerases have intrinsic RdRp activities. However, it is important to emphasize that biochemical evidence alone is insufficient to pinpoint the authentic polymerase for viroid replication in plants based on those reports.

Early investigation showed that a low concentration of α-amanitin specific for inhibiting Pol II activity can inhibit PSTVd replication in tomato protoplasts, suggesting the involvement of Pol II in PSTVd replication [106]. Subsequently, evidence indicated that Pol II can transcribe (+) PSTVd template *in vitro*, a process that can also be inhibited by α-amanitin [59]. Furthermore, Pol II can bind PSTVd and CEVd RNA *in vivo* [62,107]. Additionally, Pol II preferably binds with circular (+) genome *in vivo*, rather than linear (+) PSTVd [62], and loses transcription activity when using a linear (+) PSTVd template [108]. Lastly, based on the sensitivity to α-amanitin, other members of *Pospiviroidae* were found to use Pol II for replication, including cucumber pale fruit viroid and hop stunt viroid [2]. Therefore, Pol II is well accepted as the authentic enzyme for the replication of viroids in the family *Pospiviroidae*.

PSTVd uses its loop E region to directly bind with RPL5 and inhibits splicing regulation, favoring the accumulating of TFIIIA-7ZF. Overexpression of RPL5 suppresses the accumulation of PSTVd and TFIIIA-7ZF [70].

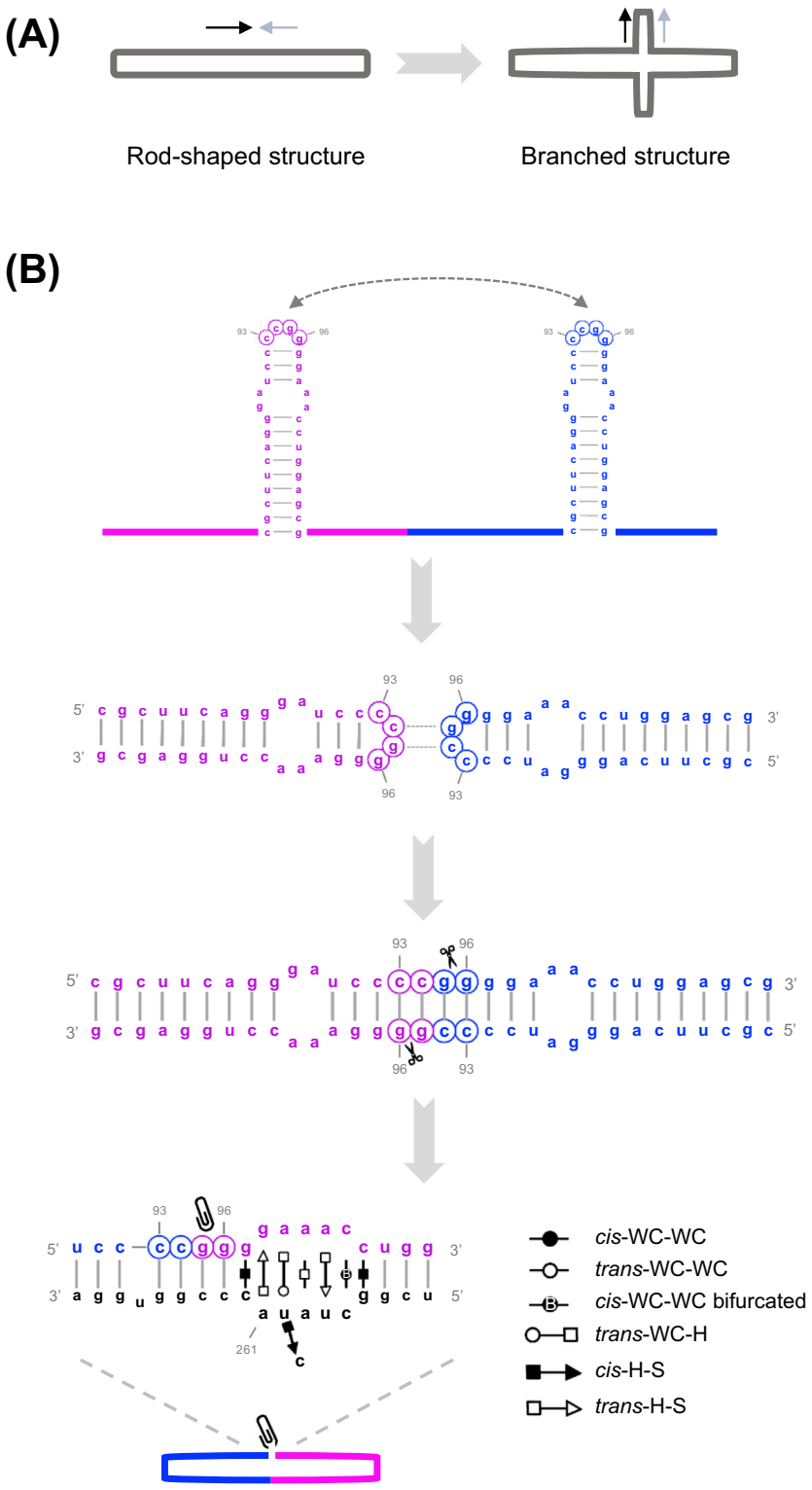
For a long time, the enzyme recognizing (–) viroid RNA templates for transcription was less clear compared with the transcription using circular (+) viroid RNA templates [2,63]. Recently, Pol II was found to bind directly to the (–) PSTVd dimer as templates to catalyze transcription (Figure 1, step c) [71]. Interestingly, Pol II transcription on the (–) dimer always initiates from the terminal region but not from the same sequence in the middle of the dimer template, as indicated by the products with a similar size of dimer. It implies a mechanism recognizing the correct initiation site. TFIIIA-7ZF also enhances Pol II activity on the (–) PSTVd template. Through functional mutagenesis, zinc-finger domains 1, 2, and 3 of TFIIIA-7ZF were found to be critical for RNA template binding, and zinc-finger domain 5 appears to mediate interaction with Pol II [71]. Most surprisingly, the Pol II complex on the (–) PSTVd template, termed remodeled Pol II, contains only six or seven subunits, which is in stark contrast to the canonical 12-subunit Pol II complex [71].

The remolded Pol II complex remains active and highly efficient in transcribing (–) PSTVd templates despite missing subunits Rpb4 (the fourth largest subunit of RNA polymerase II), Rpb5, Rpb6, Rpb7, and Rpb9. Whether Rpb12 is absent remains unknown, because this subunit was filtered out of the mass spectrometry samples due to small size. Interestingly, the absence of Rpb9, which is critical for polymerase fidelity, may explain the high error rates of viroid replication [71]. Moreover, critical general transcription factors for DNA-dependent transcription, such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH, and TFIIS, are all absent in the active transcription complex on the (–) PSTVd RNA template, suggesting distinct mechanisms used for DNA-dependent and RNA-dependent transcription [71]. However, it awaits verification whether such a remodeled Pol II functions in plants. Nevertheless, some evidence suggests that heterogeneity of Pol II complexes function in cells regulating diverse gene expressions [72]. Studying the functional organization of Pol II for viroid transcription may help to gain a comprehensive understanding of the deeply conserved transcription machinery and potentially lead to the discovery of endogenous RNA substrates of Pol II.

Viroid processing and shuttling within the nucleus

After transcription, concatemeric (+) viroid RNAs are cleaved into unit-length copies and circularized to produce progeny. These processing steps rely heavily on conformational alterations of viroid RNA structures. During processing the concatemers, the upper strand of central conserved region (CCR) in (+) viroid RNAs and the surrounding nucleotides of CCR form a conserved hairpin I (HPI) structure *in vivo* [73]. Two consecutive HPI in concatemers form a kissing-loop that contains a double-stranded region (Figure 3). This double-stranded region is cleaved by a cellular RNase III-like enzyme to yield an intermediate that contains two-nucleotide overhangs, 5′-phosphomonoester and 3′-hydroxyl termini [73]. There are seven RNase III-like nucleases in *Arabidopsis*, including four dicer-like proteins and three RNase III-like proteins. Future investigation is needed to identify the authentic enzyme for cleavage.

After cleavage, there is a structural switch from the HPI structure to the loop E structure in some members of *Pospiviroidae* (Figure 3). The formation of loop E structure is critical for ligation [73]. DNA ligase 1 (LIG1) was found to catalyze the circularization of multiple viroids [74]. Downregulation of LIG1 leads to a reduction in the accumulation of circular PSTVd in plants. Therefore, LIG1 is the authentic enzyme for circularizing nuclear-replicating viroids [74]. But there are remaining questions. Loop E motif is only conserved in a subset of viroids of *Pospiviroidae*. In addition, LIG1 exhibits a much weaker activity for *in vitro* ligation of ASSVd [74]. Is there any additional mechanism to enhance LIG1 activity for some viroids?



Trends in Microbiology

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Box 3. Cellular machinery for nuclear export

In general, exportin family members facilitate RNA–protein complexes (RNPs) to cross the nuclear pore complex. Chromosomal region maintenance 1 (CRM1), also known as exportin 1 (Xpo1), is a major mediator of nuclear export for RNAs, such as snRNAs, rRNAs, and some viral RNAs [77,78]. mRNAs can use either CRM1- or Nxf1-Nxt1 heterodimer-mediated pathways for nuclear export [77], while tRNAs and miRNAs use exportin t (Exp-t) and exportin 5 (Exp5) for nuclear export, respectively [77,78,109]. In some cases, miRNAs can also use the CRM1-based pathway for nuclear export in mammalian cells [110].

To achieve specificity in selecting RNA for nuclear export, various adapter proteins are needed for RNPs to interact with exportin proteins. For instance, free 5S rRNA uses TFIIIA as an adaptor protein to interact with CRM1 for nuclear export, while 5S rRNA in the ribosomal large subunit uses Nmd-3 as the adaptor protein to interact with CRM1 [78]. Adaptor proteins recognize specific RNA structures, such as the loop E motif in 5S rRNA that is recognized by TFIIIA [111], or interact with specific proteins in the RNP such as PHAX binding with cap-binding complex in RNPs containing snRNAs [112]. In addition, RNP–adaptor–exportin complexes rely on Ran cycles for nuclear export [77,78].

The site for viroid RNA processing within the nucleus is unknown. Previous studies have shown that (–) PSTVd intermediates remain in the nucleoplasm, while the (+) PSTVd RNA is in both the nucleoplasm and the nucleolus [75]. However, the fluorescence *in situ* hybridization (FISH) assay used in the study cannot distinguish between circular (+) PSTVd and linear (+) PSTVd. Therefore, it is possible that the cleavage and/or ligation steps may occur in either the nucleoplasm or the nucleolus. It is also possible that (+) intermediates travel into the nucleolus for cleavage and circularization (Figure 1, step d). Future investigation is necessary to determine the processing sites.

Understanding why and how (+) PSTVd is selectively imported into the nucleolus is significant because it may unravel principles for RNA targeting to the nucleolus. The distinct subcellular localization patterns of (+) and (–) PSTVd suggests the existence of selection mechanisms for nucleolar import [75]. As viroids often rely on specific RNA motifs for function, there may be a structural signal that is responsible for nucleolus import.

Possible nuclear export pathways for viroid RNAs

After replication, viroid progeny exits the nucleus and spreads into neighboring cells, which is not well understood. The nuclear export of RNAs has been well studied in other systems (Box 3). Despite using Pol II for replication in the nucleus, viroids do not possess a 5′ guanosine cap nor contain any intron [76], which are critical features for nuclear export of mRNAs and snRNAs [77,78]. Therefore, it is less likely for viroid RNAs to follow the same pathways used by snRNAs or mRNAs for nuclear export. Since the majority of (+) viroid RNAs are localized in nucleoli [75,79], one possibility is that they exploit the nuclear export pathway used by 5S rRNA/TFIIIA that initiates in nucleoli [76]. In support of this view, the canonical TFIIIA protein (i.e., TFIIIA-9ZF) interacts only with (+) PSTVd but not (–) PSTVd *in vivo* [62]. Nevertheless, further investigation is needed to understand the machinery for viroid nuclear export.

Chloroplastic viroids

It has been known for a long time that nuclear-encoded tRNAs can enter chloroplasts, contributing to translation [80]. Other than that, very few RNAs were shown to be present in chloroplasts, except an eIF4E mRNA [81] and viroids of the family *Avsunviroidae* [2,3,23]. How viroids are imported into chloroplasts remains unknown. Sequences derived from eggplant latent viroid

Figure 3. Potato spindle tuber viroid (PSTVd) structural dynamics during cleavage and ligation. (A) The change from the rod-shaped secondary structure to forming the hairpin I (HPI) structure. Arrow lines depict the regions for HPI formation. (B) Two adjacent HPI structures in (+) multimeric intermediates form interactions. Magenta and blue colors depict two unit-length regions. The terminal loops (nucleotides highlighted in circles) from two HPI structures interact to form a double-stranded region. Scissor signs indicate the cleavage sites. A loop E structure is formed to facilitate the ligation. The clipper sign indicates the ligation site. Noncanonical base pairings in the loop E structure are highlighted. Abbreviations and symbols are the same as explained in the legend of Figure 2.

(ELVd) can facilitate the import of a chimeric transcript from the nucleus into chloroplasts [82], which supports the existence of certain RNA motif(s) in regulating chloroplast import. Once inside chloroplasts, viroid RNAs are associated mainly with the thylakoid membrane in stroma but away from granal stacks (Figure 1), based on electron micrographs of ASBVd-infected samples [79].

Chloroplastic viroid transcription relies on nuclear-encoded plastid RNA polymerase (NEP), which is a DdRp. Tagetitoxin, a chemical that specifically inhibits NEP activity, impairs viroid replication in chloroplasts [83]. Furthermore, the localization of NEP in chloroplasts is consistent with the observed localization of ASBVd [84,85]. All chloroplastic viroids contain the hammerhead ribozyme in their (+) and (–) RNAs. A chloroplastic protein, *Persea americana* RNA binding protein 33 (PARBP33), interacts with viroid RNAs to enhance ribozyme activity [86]. After cleavage, chloroplastic tRNA ligase is exploited to ligate the linear intermediates into circular RNAs [87]. A tRNA anticodon structure-like motif in ELVd is critical to engage chloroplastic tRNA ligase for viroid ligation [88]. Whether additional factors are needed for viroid replication in chloroplasts deserves future investigations. For instance, elongation factor 1-alpha (eEF1A) has been shown to interact with PLMVd RNA, which may confer biological function during PLMVd replication [89].

Some observations suggest that members of *Avsunviroidae* may shuttle between the nucleus and chloroplasts. Early observations showed that both ASBVd and PLMVd can be detected, in very low amounts, in the nuclear fraction [90,91]. However, these early investigations cannot rule out possible contamination during fractionation. In a more recent analysis, ELVd sequences were embedded in the potato IV2 intron to disrupt the coding region of GFP mRNA. The appearance of green fluorescence was used to indicate the intron removal, which is presumed to occur in the nucleus [92]. However, some introns can be removed inside chloroplasts relying on ribozyme activity [93], which is also possessed by ELVd. Furthermore, emerging evidence supports that introns can be removed in the cytoplasm in plants [94–96]. In contrast to this view of chloroplast–nucleus shuttling, direct injection of fluorescein-labeled ASBVd or PLMVd transcripts failed to accumulate in the nucleus [36]. Therefore, whether chloroplastic viroids enter the nucleus during their infection remains unresolved.

Viroid spreading in plants

Viroid progeny in the cytoplasm moves to neighboring cells via plasmodesmata [97]. The detailed mechanism underlying the cell-to-cell movement of viroid is unknown. A recent study showed that Exp5 mediates cell-to-cell movement of miRNAs in plants [98]. Whether viroids also exploit Exp5 for cell-to-cell movement deserves future investigations.

To achieve systemic trafficking using the phloem system, viroids need to traffic through diverse tissues, such as the epidermis, palisade and spongy mesophylls, and bundle sheath cells [42]. Using PSTVd as a model, studies found that a set of RNA motifs mediate the RNA trafficking across different cellular boundaries [42]. In PSTVd RNA genome, loop 27, a UNCG-like motif, regulates RNA movement from epidermis to palisade mesophyll cells. Loops 6 and 19, two distinct loop motifs, regulate trafficking from palisade to spongy mesophyll cells. Loop 7, a *cis*-WC–WC base pairing with a water insertion, regulates RNA loading from bundle sheath to phloem. In addition, a bipartite motif consisting of five nucleotides at two discrete regions regulates the exiting of bundle sheath in systemic leaves. These RNA motifs likely interact with diverse cellular factors at distinct cellular boundaries, illustrating the complexity of regulating RNA systemic trafficking in plants [42]. Once viroids enter the phloem, their spreading is facilitated by PP2 [99].

Concluding remarks and future perspectives

As a class of noncoding RNAs, viroids rely heavily on their RNA motifs to traffic and replicate in plants. Those functional motifs are more conserved in structure than in primary sequences,

Outstanding questions

What are the structural elements regulating the intracellular trafficking of viroids, such as chloroplast import and export, nucleolar import, and the nuclear export? In addition, what are the cognate protein factors regulating these processes?

What are the structural elements regulating the cell-to-cell movement of viroids? Is there any difference between members of *Avsunviroidae* and *Pospiviroidae*? What are the protein factors regulating cell-to-cell movement?

How to exploit those viroid RNA motifs to control other RNA's localization in cells?

Viroids adopt alternative structures during replication and processing. How are these structural dynamics achieved and regulated?

Viroids harness a group of cellular proteins during infection. Many of these cellular proteins have critical functions in cells. Does viroid occupation of those cellular proteins interfere with normal cellular process, thereby contributing to the pathogenesis?

What are the biogenesis processes and functions of those newly identified viroid-like RNAs? How do they exert biological functions?

Multiple functional motifs have been unraveled in viroid RNAs. Some of them have known cognate protein factors for function. How to design small molecules to target these RNA motifs and interfere with their functions to block viroid infection?

constraining viroid evolution [100]. Moreover, increasing evidence supports the role of viroid structural alterations in specific processes, such as cleavage and ligation. Studying the dynamics of viroid structures in distinct cellular environments will lead to a deeper understanding of RNA structure–function relationships (see Outstanding questions).

Viroids contain critical information that enables them to localize to different subcellular compartments. Deciphering those elements will provide opportunities to manipulate subcellular localizations of various RNAs, facilitating the development of RNA-base biotechnologies.

With the exponential increase of identified viroid-like RNAs, future investigations are needed to elucidate their functions and biogenesis. Studies on viroids provide key insights into RNA–RNA amplification mechanisms and RNA structure–function relationships, which can help in understanding the biological significance of the increasing numbers for viroid-like RNAs and other noncoding RNAs.

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Declaration of interests

No interests are declared.

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